

## MACROCYCLIC HEXAPEPTIDE ANALOGUES OF THE THROMBIN RECEPTOR (PAR-1) ACTIVATION MOTIF SFLLRN

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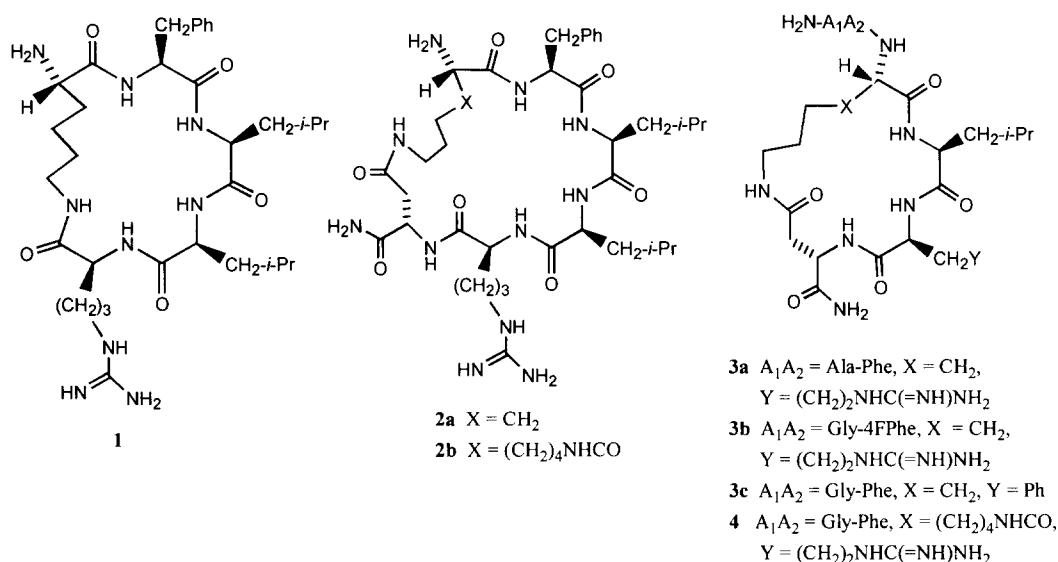
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**Abstract:** The thrombin receptor (PAR-1) is activated by  $\alpha$ -thrombin to stimulate various cell types, including platelets, through the tethered-ligand sequence SFLLRN. Macrocyclic peptide analogues of SFLLRN were synthesized and evaluated *in vitro*. In general, the compounds were much less potent in inducing platelet aggregation relative to SFLLRN-NH<sub>2</sub> and did not act as antagonists of  $\alpha$ -thrombin. Derivative **3c** was the most potent macrocycle in activating PAR-1, with an EC<sub>50</sub> of 24  $\mu$ M. © 1999 Elsevier Science Ltd. All rights reserved.

$\alpha$ -Thrombin, a trypsin-like serine protease centrally involved in hemostasis, also promotes diverse cellular responses such as platelet aggregation, lymphocyte mitosis, monocyte chemotaxis, and endothelial cell proliferation.<sup>1</sup> These actions are mediated by specific receptors on the cell surface, which eluded identification for many years. However, in 1991 Coughlin and coworkers cloned and expressed the first example of a thrombin receptor (protease-activated receptor 1; PAR-1) and identified it as a member of the vast G-protein coupled receptor (GPCR) superfamily.<sup>2</sup> Thrombin proteolytically cleaves the lengthy N-terminal extracellular domain of PAR-1 at the Arg-41/Ser-42 peptide bond to reveal a new N-terminus containing the sequence SFLLRN, which apparently serves as a “tethered ligand.” Significantly, synthetic SFLLRN-NH<sub>2</sub> (TRAP-6), and peptide analogues thereof, are capable of activating PAR-1 in a manner similar to thrombin, albeit at higher concentrations (50–1000 times greater).<sup>2</sup> Other protease-activated receptors with close homology to the original thrombin receptor, namely PAR-2,<sup>3,4</sup> PAR-3,<sup>5</sup> and PAR-4,<sup>6</sup> have recently been cloned as well, and these possess their own agonist peptide motifs.

SFLLRN-based agonist peptides have received considerable structure-activity attention in terms of platelet activation studies.<sup>1,d,e,7–9</sup> The minimum structural requirements for the PAR-1 peptides (thrombin receptor-activating peptides; TRAPs) are, generally, at least an N-terminal pentapeptide with a free amino group at position (P) 1, a small side chain at P1, an aromatic residue at P2 (with agonist potency enhanced ca. four-fold by *p*-F-Phe substitution<sup>10</sup>), and a basic or aromatic residue at P5. A large hydrophobic amino acid at P4 is important as well, but widely varied substitution is tolerated at P3. We<sup>11</sup> and others<sup>12</sup> have also been interested in the structural properties of the peptide backbone, relative to function, to gain an understanding of possible bioactive conformations. Cyclic peptides are expected to adopt folded conformations, such as  $\beta$ - or  $\gamma$ -turns, which are fixed by intramolecular hydrogen bonding. Hence, we sought to investigate macrocyclic

analogues of SFLLRN and ascertain the effect of conformational constraint on PAR-1 agonist activity in the form of platelet activation, as well as possible PAR-1 antagonist activity. During the course of our work, Matsoukas et al.<sup>12b</sup> reported three macrocyclic analogues of SFLLR with a cyclization linkage between the P1 side chain and the C-terminus and found that **1** has nearly the same potency as SFLLR-NH<sub>2</sub> in inducing contractions of gastric smooth muscle. We now report our studies of a variety of macrocyclic hexapeptide analogues of SFLLRN with a cyclization linkage between the P1/P6 (**2**) or the P3/P6 (**3** and **4**) side chains.

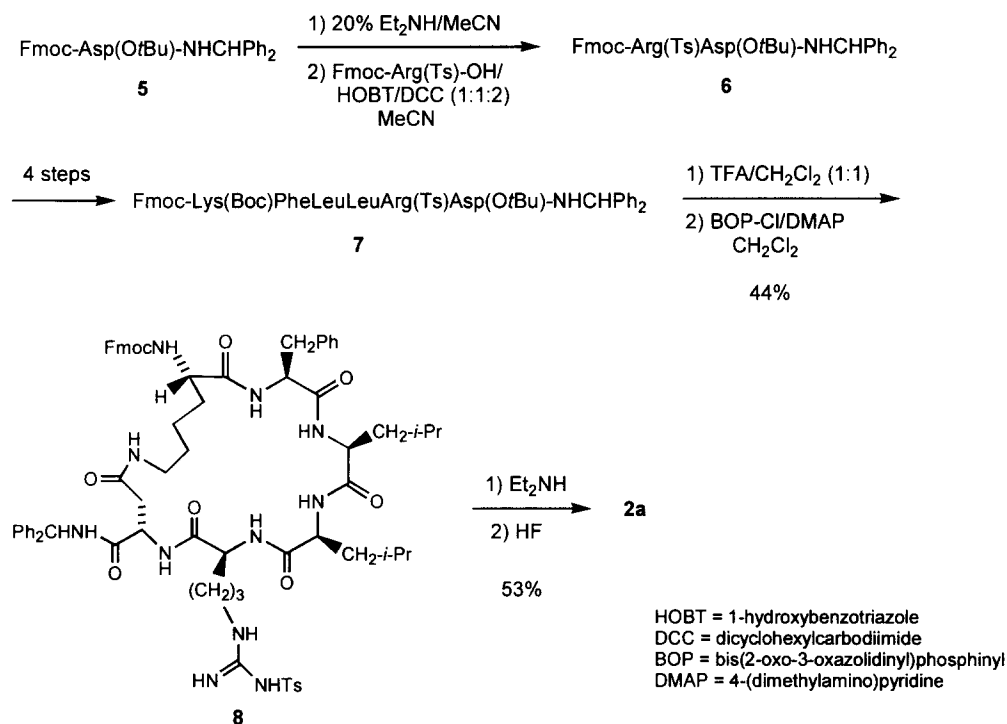


**Synthesis.** Cyclic peptides **2a**, **2b**, **3a–3c**, and **4** were synthesized by using a classical solution-phase Fmoc protocol<sup>13</sup> exemplified in Scheme 1. Fmoc removal from **5** was accomplished with 20% Et<sub>2</sub>NH in acetonitrile and the resultant amine was coupled with protected arginine, as shown, to give a quantitative yield of dipeptide **6**. The dipeptide was elaborated by using the same deprotection/coupling procedure to give hexapeptide **7**. The Boc and *t*-Bu ester groups were removed with 50% CF<sub>3</sub>CO<sub>2</sub>H (TFA) in CH<sub>2</sub>Cl<sub>2</sub> and, after evaporation in vacuo, the amino acid was cyclized by using BOP-Cl/DMAP<sup>14</sup> to afford protected cyclic peptide **8**. Deprotection of the acid-labile groups was performed with HF/anisole at 0 °C for 3.5 h and product **2a** was purified by reverse-phase HPLC. Compounds prepared analogously are shown in Table 1.

To more rapidly obtain compounds **3** in which A<sub>1</sub> and A<sub>2</sub> are widely varied, we resorted to solid-phase synthesis of a mini-library. This entailed preparation of the dipeptide A<sub>1</sub>A<sub>2</sub> on a trityl resin, coupling with the appropriate cyclic tetrapeptide, prepared as above, and cleavage from the solid support with TFA (Scheme 2). Thus, trityl chloride resin (Advanced Chemtech) was reacted with the allyl ester of β-alanine and *i*-Pr<sub>2</sub>NEt in DMF<sup>15</sup> to give the amino acid-functionalized resin, **9**. Palladium-catalyzed deprotection of the allyl ester<sup>16</sup> and coupling with phenylalanine allyl ester gave **10**, which was deprotected to afford dipeptide **11**. Diisopropylcarbodiimide (DIC) coupling of **11** with cyclic tetrapeptide **12**, prepared according to the route in Scheme 1, gave the resin-bound target, which was cleaved from the support with 50% TFA in

$\text{CH}_2\text{Cl}_2$ . Concentration in vacuo and trituration with ether provided **3p**. Similarly, **3c–3z** were prepared, with arginine being protected by a 2,4,6-trimethylbenzenesulfonyl (Mts) group that was readily removed with triflic acid/TFA/anisole during cleavage from the resin.

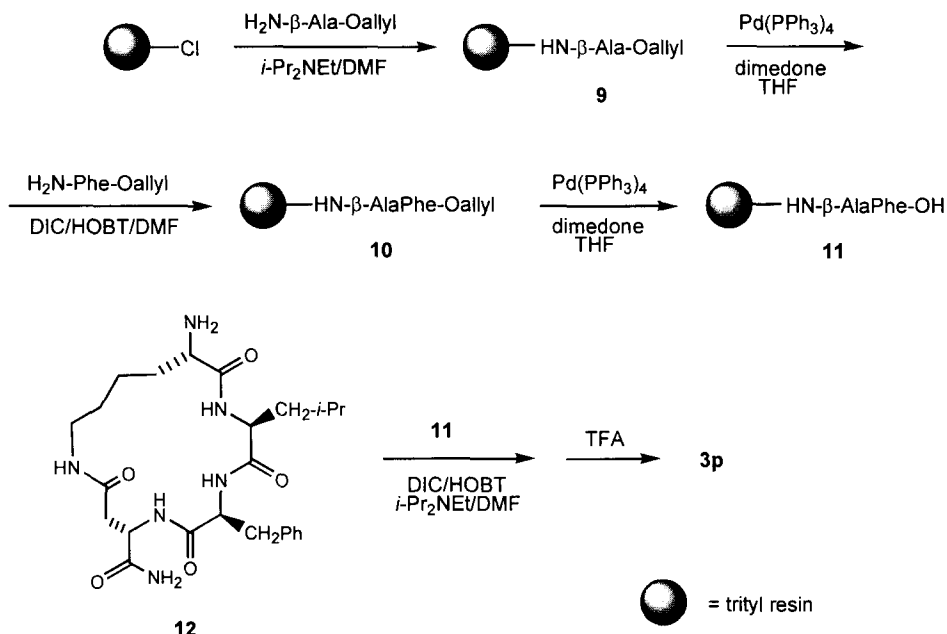
Scheme 1

Table 1. Chemical Properties<sup>a</sup>

| compd     | formula                                                                                                                      | mp, °C <sup>b</sup> | $[\alpha]_D^{25}$ (c, MeOH) |
|-----------|------------------------------------------------------------------------------------------------------------------------------|---------------------|-----------------------------|
| <b>2a</b> | $\text{C}_{37}\text{H}_{61}\text{N}_{11}\text{O}_7 \cdot 2.5 \text{ CF}_3\text{CO}_2\text{H} \cdot 2.5 \text{ H}_2\text{O}$  | 182–190             | –6.9° (0.65)                |
| <b>2b</b> | $\text{C}_{41}\text{H}_{68}\text{N}_{12}\text{O}_8 \cdot 2.5 \text{ CF}_3\text{CO}_2\text{H} \cdot 4.0 \text{ H}_2\text{O}$  | 142–150             | –30.5° (0.73)               |
| <b>3a</b> | $\text{C}_{34}\text{H}_{55}\text{N}_{11}\text{O}_7 \cdot 2.5 \text{ CF}_3\text{CO}_2\text{H} \cdot 3.0 \text{ H}_2\text{O}$  | 225–235             | +2.6° (0.51)                |
| <b>3b</b> | $\text{C}_{33}\text{H}_{52}\text{FN}_{11}\text{O}_7 \cdot 2.2 \text{ CF}_3\text{CO}_2\text{H} \cdot 4.2 \text{ H}_2\text{O}$ | >250                | +2.3° (0.82)                |
| <b>3c</b> | $\text{C}_{36}\text{H}_{50}\text{N}_8\text{O}_7 \cdot 3.5 \text{ CF}_3\text{CO}_2\text{H} \cdot 3.5 \text{ H}_2\text{O}$     | >250                |                             |
| <b>3o</b> | $\text{C}_{34}\text{H}_{55}\text{N}_{11}\text{O}_7 \cdot 3.5 \text{ CF}_3\text{CO}_2\text{H} \cdot 2.0 \text{ H}_2\text{O}$  | 230–240             | –6.7° (0.73)                |
| <b>4</b>  | $\text{C}_{37}\text{H}_{60}\text{N}_{12}\text{O}_8 \cdot 3.0 \text{ CF}_3\text{CO}_2\text{H} \cdot 2.0 \text{ H}_2\text{O}$  | 225–230             | –5.8° (0.67)                |

a. These compounds were purified by reverse-phase HPLC by using 0.16% TFA in MeCN/0.20% TFA in water and lyophilized to white solids; they were characterized by mass spectrometry and high-field proton NMR. Microanalytical data (C, H, N) were within the accepted range ( $\pm 0.4\%$ ); % water was determined by Karl-Fisher analysis. b. Mp values are corrected to a set of standards.

Scheme 2



**Biological Results.** The cyclic peptides were screened for agonist and antagonist activity by using human gel-filtered platelet aggregation.<sup>11,17</sup> For inhibition of platelet aggregation  $\alpha$ -thrombin was used as the agonist (Table 2); some results were also obtained with TRAP-6 (50  $\mu\text{M}$ ) as the agonist (**2b**, 6%; **3a**, 6%, **3b**, 9%; **3c**, 26%; **4**, 49%). The P1/P6 cyclic peptides **2a** and **2b** showed very little agonist activity, which might be attributed to the larger ring size of 23 or 28 atoms in comparison to **1** with 19 atoms. Although **2b** showed modest antagonist activity with an  $\text{IC}_{50}$  of ca. 50  $\mu\text{M}$ , whereas **2a** was devoid of antagonist activity, this is probably not solely related to PAR-1 since **2b** inhibited thrombin to a modest degree (26% inhibition at 50  $\mu\text{M}$ ). The P3/P6 cyclic tetrapeptides **3a** and **3b**, with a smaller 17-membered ring, exhibited improved, albeit modest, agonist activity ( $\text{EC}_{50}$  of ca. 60  $\mu\text{M}$ ). Given the agonist activity of **3a** and **3b**, we conducted a study with variations at the  $\text{A}_1/\text{A}_2$  positions of **3**. Changing  $\text{A}_1$  and  $\text{A}_2$  while retaining the arginine, as in **3d-3o**, led to a loss of agonist activity. However, when Arg was replaced with Phe, as in **3c**, agonist activity was increased; also, **3v-3x** had reasonable agonist activity. Insertion of a GABA unit into the ring of inactive **3g** gave 22-membered macrocycle **4**, which exhibited modest agonist ( $\text{EC}_{50} = 60 \mu\text{M}$ ) and antagonist (44% inhibition at 50  $\mu\text{M}$ ) activity. This activity for **3g** and **4** may be attributed to increased flexibility of the larger ring, which can orient the critical side chains into the proper spatial arrangement for binding to the receptor.

In summary, 17-membered P3/P6 macrocycles **3a** and **3b** showed modest agonist activity ( $\text{EC}_{50} = 60 \mu\text{M}$ ), albeit ca. 200 times less than SFLLRN-NH<sub>2</sub>. Through structural variations of this macrocyclic tetrapeptide, we found that replacement of Arg with Phe (viz. **3c**) can afford reasonable agonist activity ( $\text{EC}_{50} = 24 \mu\text{M}$ ). Increasing the ring size of inactive **3g** by insertion of a GABA unit, between the Lys and Asp side chains, gave **4**, which exhibited improved, albeit modest, agonist activity.

**Table 2. Biological Data<sup>a</sup>**

| cmpd             | A <sub>1</sub> A <sub>2</sub> | Y                                                       | agonist activ. <sup>b</sup><br>EC <sub>50</sub> (% aggr.) | antagonist activ. <sup>c</sup><br>% inh. vs thrombin |
|------------------|-------------------------------|---------------------------------------------------------|-----------------------------------------------------------|------------------------------------------------------|
| <b>2a</b>        | --                            | --                                                      | (6)                                                       | 3                                                    |
| <b>2b</b>        | --                            | --                                                      | (5)                                                       | 52                                                   |
| <b>3a</b>        | Ala-Phe                       | (CH <sub>2</sub> ) <sub>2</sub> NHC(=NH)NH <sub>2</sub> | 66                                                        | 2                                                    |
| <b>3b</b>        | Gly-4FPhe                     | (CH <sub>2</sub> ) <sub>2</sub> NHC(=NH)NH <sub>2</sub> | 60                                                        | 14                                                   |
| <b>3c</b>        | Gly-Phe                       | Ph                                                      | 24                                                        | 23                                                   |
| <b>3d</b>        | β-Ala-Phe                     | (CH <sub>2</sub> ) <sub>2</sub> NHC(=NH)NH <sub>2</sub> | (7)                                                       | 3                                                    |
| <b>3e</b>        | 4-Abu-Phe                     | (CH <sub>2</sub> ) <sub>2</sub> NHC(=NH)NH <sub>2</sub> | (6)                                                       | 6                                                    |
| <b>3f</b>        | Ser-Phe                       | (CH <sub>2</sub> ) <sub>2</sub> NHC(=NH)NH <sub>2</sub> | (16)                                                      | 13                                                   |
| <b>3g</b>        | Gly-Phe                       | (CH <sub>2</sub> ) <sub>2</sub> NHC(=NH)NH <sub>2</sub> | (15)                                                      | 9                                                    |
| <b>3h</b>        | β-Ala-3FPhe                   | (CH <sub>2</sub> ) <sub>2</sub> NHC(=NH)NH <sub>2</sub> | (7)                                                       | 3                                                    |
| <b>3i</b>        | 4-Abu-3FPhe                   | (CH <sub>2</sub> ) <sub>2</sub> NHC(=NH)NH <sub>2</sub> | (6)                                                       | 2                                                    |
| <b>3j</b>        | Ser-3FPhe                     | (CH <sub>2</sub> ) <sub>2</sub> NHC(=NH)NH <sub>2</sub> | (12)                                                      | 5                                                    |
| <b>3k</b>        | Gly-3FPhe                     | (CH <sub>2</sub> ) <sub>2</sub> NHC(=NH)NH <sub>2</sub> | (6)                                                       | 10                                                   |
| <b>3l</b>        | β-Ala-hPhe                    | (CH <sub>2</sub> ) <sub>2</sub> NHC(=NH)NH <sub>2</sub> | (7)                                                       | 1                                                    |
| <b>3m</b>        | 4-Abu-hPhe                    | (CH <sub>2</sub> ) <sub>2</sub> NHC(=NH)NH <sub>2</sub> | (8)                                                       | 2                                                    |
| <b>3n</b>        | Ser-hPhe                      | (CH <sub>2</sub> ) <sub>2</sub> NHC(=NH)NH <sub>2</sub> | (16)                                                      | 5                                                    |
| <b>3o</b>        | Gly-hPhe                      | (CH <sub>2</sub> ) <sub>2</sub> NHC(=NH)NH <sub>2</sub> | (5)                                                       | 2                                                    |
| <b>3p</b>        | β-Ala-Phe                     | Ph                                                      | NA                                                        | 11                                                   |
| <b>3q</b>        | 4-Abu-Phe                     | Ph                                                      | NA                                                        | 14                                                   |
| <b>3r</b>        | Ser-Phe                       | Ph                                                      | NA                                                        | 12                                                   |
| <b>3s</b>        | β-Ala-3FPhe                   | Ph                                                      | NA                                                        | 24                                                   |
| <b>3t</b>        | 4-Abu-3FPhe                   | Ph                                                      | NA                                                        | 27                                                   |
| <b>3u</b>        | Ser-3FPhe                     | Ph                                                      | NA                                                        | 23                                                   |
| <b>3v</b>        | Gly-3FPhe                     | Ph                                                      | 63                                                        | 11                                                   |
| <b>3w</b>        | β-Ala-hPhe                    | Ph                                                      | 43                                                        | 16                                                   |
| <b>3x</b>        | 4-Abu-hPhe                    | Ph                                                      | 37                                                        | 15                                                   |
| <b>3y</b>        | Ser-hPhe                      | Ph                                                      | NA                                                        | 2                                                    |
| <b>3z</b>        | Gly-hPhe                      | Ph                                                      | NA                                                        | 1                                                    |
| <b>4</b>         | Gly-Phe                       | (CH <sub>2</sub> ) <sub>2</sub> NHC(=NH)NH <sub>2</sub> | 30                                                        | 44                                                   |
| std <sup>d</sup> |                               |                                                         | 0.80                                                      | 0                                                    |

a. Abbreviations: 4-Abu = 4-aminobutyl; hPhe = homophenylalanine. b. Activation of human platelet aggregation (gel-filtered platelets), expressed as an EC<sub>50</sub> value in μM or as percent aggregation induced by the test compound at 50 μM (in parentheses), performed as reported in ref 17. NA indicates no activity at 100 μM. c. Inhibition of human platelet aggregation (gel-filtered platelets) induced by human α-thrombin, expressed as percent inhibition at 50 μM (ref 17). d. Reference standard, SFLLRN-NH<sub>2</sub> (TRAP-6).

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